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EPIDEMIOLOGY AND SURVEILLANCE

Emergence of Various NDM-Type-Metallo-β-Lactamase-Producing *Escherichia coli* Clinical Isolates in Nepal

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ABSTRACT Of 250 clinical isolates of *Escherichia coli* obtained in Nepal, 38 were carbapenem resistant, with MICs of imipenem or meropenem of ≥4 μg/ml. All 38 isolates harbored the following bla_{NDM}s: bla_{NDM-1}, bla_{NDM-3}, bla_{NDM-4}, bla_{NDM-5}, bla_{NDM-7}, bla_{NDM-12}, and bla_{NDM-13}. Most of these isolates also harbored the 16S rRNA methylase gene(s) *armA*, *rmtB*, and/or *rmtC*.

KEYWORDS NDM-type metallo-β-lactamase, carbapenem-resistant *Escherichia coli*, molecular epidemiology

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Metallo-β-lactamases (MBLs) can confer resistance to carbapenems, reducing their susceptibility to carbapenems, as well as to cephalosporins and penicillins but not to monobactams (1). New Delhi metallo-β-lactamase-1 (NDM-1) was initially isolated in 2008 from *Klebsiella pneumoniae* and *Escherichia coli* strains originating in India (2). To date, 16 NDM variants have been identified (ftp://ftp.ncbi.nlm.nih.gov/pathogen/betalactamases/Allele.tab). Since the initial observation, NDM-producing *Enterobacteriaceae* strains have been isolated in various parts of the world, including Australia, Bangladesh, Belgium, Canada, France, India, Kenya, Japan, Nepal, the Netherlands, New Zealand, Pakistan, Singapore, Taiwan, and the United States (3–6). This study analyzed the molecular epidemiology of carbapenem-resistant *E. coli* isolates obtained from a university hospital in Nepal.

A total of 250 consecutive nonrepetitive clinical isolates of *E. coli* were obtained from 250 patients hospitalized between December 2013 and December 2014 at a university hospital in Kathmandu, Nepal. These bacterial isolates were collected from urine (n = 92), pus (n = 65), sputum (n = 36), catheter specimens (n = 35), blood (n = 9), bile (n = 5), body fluid (n = 4), and other specimens (n = 4). Species identification was confirmed by 16S rRNA sequencing (7). Antimicrobial susceptibility to amikacin, arbekacin, ceftazidime, ciprofloxacin, colistin, imipenem, meropenem, and tigecycline was tested by the microdilution method according to the guidelines of the Clinical and Laboratory Standards Institute (8). Carbapenem-resistant *E. coli* isolates were defined as having imipenem or meropenem MICs of ≥4 μg/ml. The whole genomes of all carbapenem-resistant *E. coli* isolates were extracted using DNeasy blood and tissue kits (Qiagen, Tokyo, Japan) and sequenced with a MiSeq sequencer (Illumina, San Diego, CA). The raw reads were assembled using CLC Genomics Workbench version 8.0.2 (CLC bio, Tokyo, Japan). A summary of the assembly is shown in Table S1 in the supplemental material. The whole-genome sequences of all 38 isolates were deposited in GenBank as accession no. DRA005225. To analyze the relationships among these 38 *E. coli* isolates, the complete genome of an *E. coli* sequence type 2747 (ST2747) strain (GenBank accession no. CP007394) was used as a reference, because a BLAST search showed that

TABLE 1 MIC_{90} and MIC_{50} values and percentages of antimicrobial resistance among *E. coli* clinical isolates[a]

Antimicrobial agent	Breakpoint for resistance (μg/ml)	% of strains resistant	MIC (μg/ml)		
			Range	MIC_{50}	MIC_{90}
Amikacin	≥64	87	2–>512	>512	>512
Arbekacin			1–>512	>512	>512
Ceftazidime	≥16	100	512–>512	>512	>512
Ciprofloxacin	≥4	100	8–>256	128	>512
Colistin	≥4	0	≤0.125–1	0.125	1
Imipenem	≥4	100	4–256	16	64
Meropenem	≥4	100	8–128	32	64
Tigecycline	≥4	5	≤0.125–4	2	2

[a]Thirty-eight isolates were tested. Breakpoints for antimicrobial resistance were determined according to guidelines of the Clinical and Laboratory Standards Institute. The breakpoint for tigecycline resistance was provided for *Enterobacteriaceae* by EUCAST and the U.S. Food and Drug Administration (FDA).

this strain was genetically close to the isolates tested (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). Concatenated single nucleotide polymorphism (SNP) sequences were aligned by MAFFT (http://mafft.cbrc.jp/alignment/server/). Models and parameters used for the phylogenetic analyses were computed using jModelTest 2.1.4. A maximum-likelihood phylogenetic tree was constructed from SNP alignment with PhyML 3.0 (9). The sequences of drug resistance genes, including β-lactamase-encoding genes, aminoglycoside resistance genes, and quinolone resistance genes, were determined using ResFinder 2.1 (https://cge.cbs.dtu.dk/services/ResFinder/). Multilocus sequence types (MLSTs) were deduced as described in the protocols of the University of Warwick MLST databases (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli), and clonal complexes (CC) were determined by eBURST version 3 (http://eburst.mlst.net). To determine the sizes of the plasmids harboring bla_{NDM}s, plasmid DNAs in each isolate were extracted and digested with S1 nuclease. Pulsed-field gel electrophoresis (PFGE) and Southern hybridization were performed (10).

Of the 250 *E. coli* isolates, 38 were carbapenem resistant; 9 of these were from respiratory tracts, 10 were from pus and wounds, 18 were from urinary tracts, and 1 was from other sources. According to CLSI guidelines (11), 33 (87%) of the 38 isolates were multidrug resistant (Table 1). All isolates were resistant to ceftazidime, ciprofloxacin, and meropenem, but they were susceptible to colistin. Thirty-six isolates (95%) were sensitive to tigecycline (MICs of ≤1 μg/ml), whereas for two isolates (5%), the MICs were 4 μg/ml. The 38 carbapenem-resistant *E. coli* isolates belonged to 11 different MLSTs: ST38 (3 isolates), ST94 (1 isolate), ST101 (6 isolates), ST167 (5 isolates), ST361 (2 isolates), ST405 (8 isolates), ST448 (3 isolates), ST648 (5 isolates), ST2083 (2 isolates), ST2659 (1 isolate), and ST4108 (2 isolates) (Table 2). eBURST analysis revealed that the ST38 and ST2659 isolates belonged to CC38 and that ST94, ST448, and ST2083 isolates belonged to CC94. A maximum-likelihood phylogenetic tree constructed from the 38 carbapenem-resistant *E. coli* isolates revealed three clades (Fig. 1). Clade A consisted of the isolates belonging to ST38, ST405, ST2659, and ST2747 (an *E. coli* ST2747 strain was the reference strain). Clade B comprised the isolates belonging to ST94, ST101, ST167, ST361, ST448, ST2083, and ST4108. Clade C had one isolate belonging to ST648. Clade A contained subclade CC38, which consisted of the isolates belonging to ST38 and ST2659, and clade B contained subclade CC94, which consisted of the isolates belonging to ST94, ST448, and ST2083. All 38 carbapenem-resistant *E. coli* isolates harbored bla_{NDM}s, with 16, 11, 7, 1, 1, 1, and 1 harboring bla_{NDM-1}, bla_{NDM-5}, bla_{NDM-7}, bla_{NDM-3}, bla_{NDM-4}, bla_{NDM-12}, and bla_{NDM-13}, respectively (Table 2). In addition, 30 isolates, 29 isolates, 1 isolate, and 1 isolate harbored $bla_{CTX-M-15}$, bla_{TEM-1}, $bla_{TEM-166}$, and $bla_{OXA-181}$, respectively (Table S2).

Of the 38 isolates, 33 (87%) harbored 16S rRNA methylase-encoding genes, including 14, 11, and 4 harboring *armA*, *rmtB*, and *rmtC*, respectively; 2 harboring both *armA* and *rmtB*; 2 harboring both *armA* and *rmtC*; and 30 harboring *aac(6′)-Ib-cr* (Table S2).

TABLE 2 Summary of the characteristics of the 38 carbapenem-resistant *E. coli* strains, including MLSTs and drug resistance genes[a]

MLST	No. of isolates	Clonal complex	Carbapenemase- and extended-spectrum β-lactamase-encoding gene(s)	16S rRNA methylase- and aminoglycoside acetyltransferase-encoding genes	Mutation(s) in DNA gyrase		
					GyrA	ParC	ParE
ST38	3	CC38	$bla_{\text{NDM-1}}$ (2/3), $bla_{\text{NDM-3}}$ (1/3), $bla_{\text{CTX-M-15}}$	*rmtC*, *aac(6')-1b-cr*, *aac(3)-IIa* (1/3), *aadA1*	S83L, D87N	S80I, E84G	
ST94	1	CC94	$bla_{\text{NDM-1}}$	*armA*, *aac(6')-1b-cr*, *aadA1*, *aadA2*	S83L, D87N	S80I	
ST101	6		$bla_{\text{NDM-1}}$ (3/6), $bla_{\text{NDM-5}}$ (1/6), $bla_{\text{NDM-7}}$ (1/6), $bla_{\text{NDM-13}}$ (1/6), $bla_{\text{CTX-M-15}}$ (5/6), $bla_{\text{TEM-166}}$ (1/6)	*armA* (2/6), *armA* and *rmtB* (2/6), *rmtB* (1/6), *aac(6')-1b-cr*, *aadA1* (3/6), *aadA2* (2/6)	S83L, D87N	S80I	E455D
ST167	5		$bla_{\text{NDM-5}}$, $bla_{\text{CTX-M-15}}$ (4/5), $bla_{\text{OXA-181}}$ (1/5)	*rmtB*, *aac(6')-1b-cr* (2/5), *aadA1* (1/5), *aadA2* (3/5), *aadA5* (1/5)	S83L, D87N	S80I	
ST361	2		$bla_{\text{NDM-1}}$ (1/2), $bla_{\text{NDM-5}}$ (1/2), $bla_{\text{CTX-M-15}}$	*rmtB* (1/2), *rmtC* (1/2), *aac(6')-1b* (1/2), *aadA1*, *aadA2* (1/2), *aphA6* (1/2)	S83L, D87N	S80I	
ST405	8		$bla_{\text{NDM-1}}$ (4/8), $bla_{\text{NDM-4}}$ (1/8), $bla_{\text{NDM-5}}$ (2/8), $bla_{\text{NDM-7}}$ (1/8), $bla_{\text{CTX-M-15}}$ (7/8)	*armA* (4/8), *armA* and *rmtC* (1/8), *rmtB* (2/8), *aac(6')-1b-cr* (6/8), *aadA2*, *aphA6* (2/8), *aac(3)-IIa* (1/8)	S83L, D87N	S80I	
ST448	3	CC94	$bla_{\text{NDM-1}}$ (1/3), $bla_{\text{NDM-5}}$ (1/3), $bla_{\text{NDM-12}}$ (1/3), $bla_{\text{CTX-M-15}}$ (1/3)	*armA*(1/3), *rmtB* (1/3), *aac(6')-1b-cr*, *aac(3)-1Id* (1/3), *aadA1* (1/3), *aadA2* (1/3)	S83L, D87N	S80I, E84G	
ST648	5		$bla_{\text{NDM-1}}$ (1/5), $bla_{\text{NDM-5}}$, (1/5), $bla_{\text{NDM-7}}$ (3/5), $bla_{\text{CTX-M-15}}$ (4/5)	*armA* (2/5), *armA* and *rmtC* (1/5), *rmtB* (1/5), *aac(6')-1b-cr* (4/5), *aadA1* (1/5), *aadA2* (4/5)	S83L, D87N	S80I	
ST2083	2	CC94	$bla_{\text{NDM-1}}$, $bla_{\text{CTX-M-15}}$ (1/2)	*armA*, *aac(6')-1b-cr*, *aadA1*, *aadA2*	S83L, D87N	S80I	
ST2659	1	CC38	$bla_{\text{NDM-1}}$, $bla_{\text{CTX-M-15}}$	*rmtC*, *aac(6')-1b-cr*	S83L, D87N	S80I	
ST4108	2		$bla_{\text{NDM-7}}$, $bla_{\text{CTX-M-15}}$	*armA*, *aac(6')-1b-cr*, *aadA1*, *aadA2*	S83L, D87N	S80I	

[a]Numbers in parentheses are the number of isolates with the named gene/number tested.

Sequences derived from each contig datum after assembly of the raw read data showed that 16 types of genetic structures surrounded bla_{NDM}s (Fig. 2). Of the 16 isolates harboring $bla_{\text{NDM-1}}$, 7 were type B, 5 were type A, and 1 each was type C, D, E, or F. The genomic environment surrounding $bla_{\text{NDM-3}}$ was *tnpA* $bla_{\text{NDM-3}}$ ble_{MBL} *trpF*

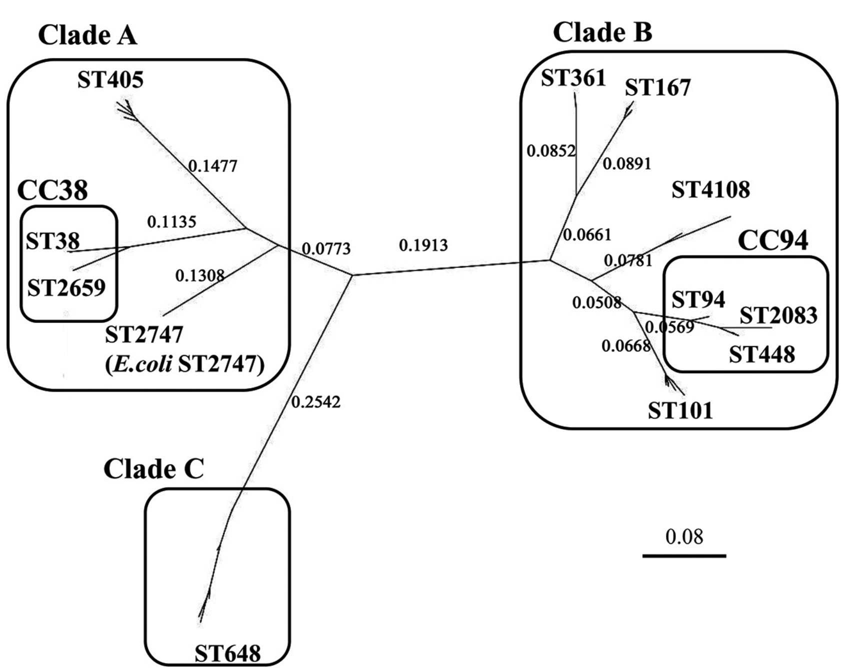

FIG 1 Molecular phylogeny of the 38 *E. coli* strains. A maximum-likelihood phylogenetic tree constructed from the 38 carbapenem-resistant isolates revealed three clades. The length of the concatemer was 244,995 bp. Clade A consisted of the isolates belonging to ST38, ST405, ST2659, and ST2747 (*E. coli* ST2747 was the reference strain). Clade B consisted of the isolates belonging to ST94, ST101, ST167, ST361, ST448, ST2083, and ST4108. Clade C consisted of only one isolate, and it belonged to ST648. Clade A contained subclade CC38, consisting of isolates belonging to ST38 and ST2659, and clade B contained subclade CC94, consisting of isolates belonging to ST94, ST448, and ST2083.

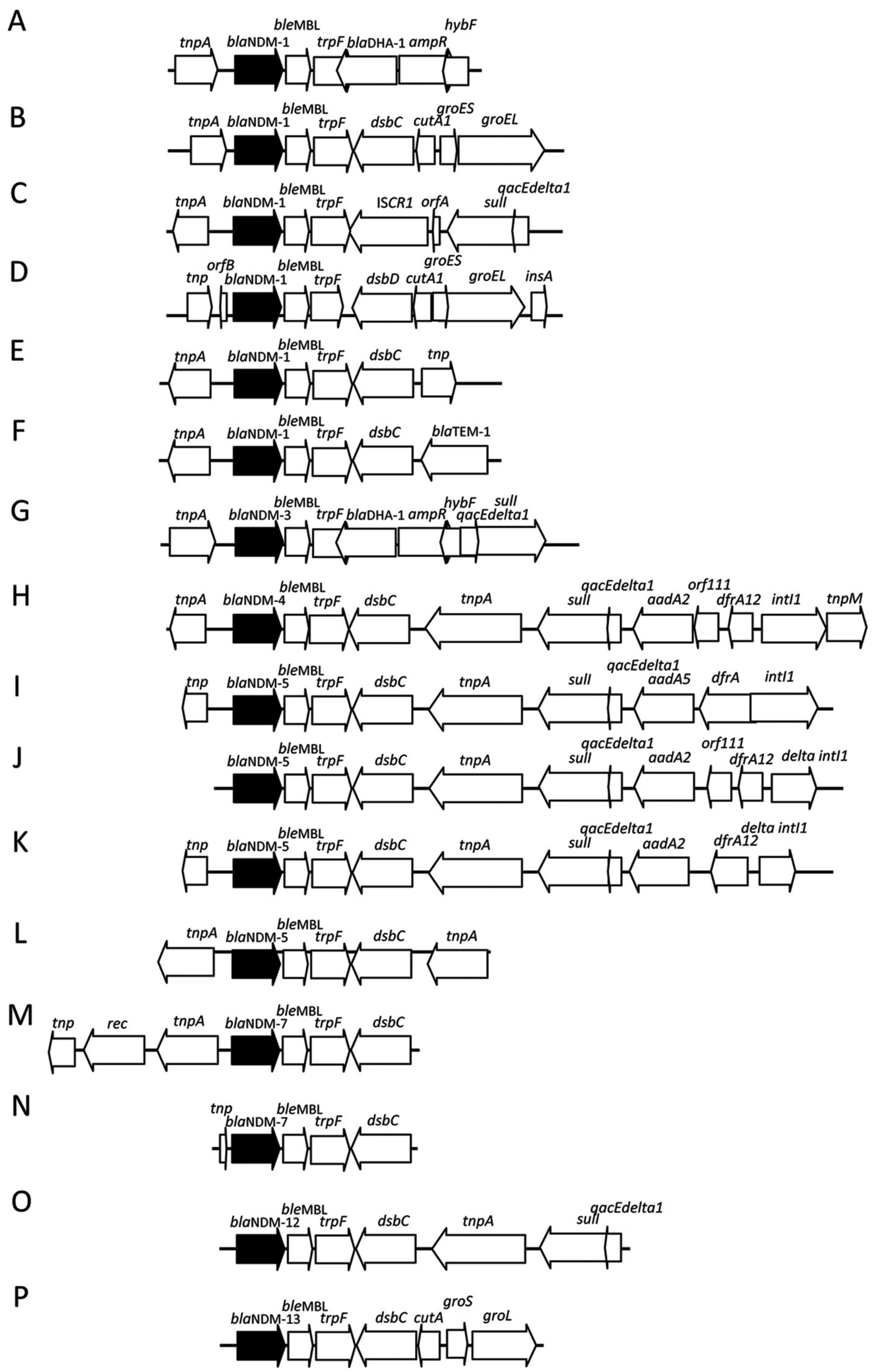

FIG 2 Structures of the genomic environments, including of $bla_{\text{NDM-1}}$ (A to F), $bla_{\text{NDM-3}}$ (G), $bla_{\text{NDM-4}}$ (H), $bla_{\text{NDM-5}}$ (I to L), $bla_{\text{NDM-7}}$ (M and N), $bla_{\text{NDM-12}}$ (O), and $bla_{\text{NDM-13}}$ (P), from sequences from each contig datum after assembling. *orfA* and *orfB*, hypothetical protein-encoding genes.

$bla_{\text{DHA-1}}$ *ampR hybF qacEdelta1 sulI* (type G in Fig. 2). This sequence showed greater than 99.9% identity with that in NDM-3-producing *E. coli* EC2 plasmid pEC2-NDM-3 in Australia (12). The genomic environment surrounding $bla_{\text{NDM-4}}$ was *tnpA* $bla_{\text{NDM-4}}$ ble_{MBL} *trpF dsbC tnpA sul1 qacEdelta1 aadA2 orf111 dfrA12 intI1 tnpM* (type H in Fig. 2). This sequence had more than 99.9% identity with that in NDM-1-producing *E. coli* GUE plasmid pGUE-NDM in India (13). The genomic environments surrounding $bla_{\text{NDM-5}}$ had

4 types of structures: types I, J, K, and L (Fig. 2). Of 11 NDM-5 producers, 6 were type I, 3 were type J, and 2 each were type K or L. The genomic environment surrounding $bla_{\text{NDM-7}}$ had two types of structures, M and N (Fig. 2). The structure in type M was unique, whereas the structure in type N was identical to that in the NDM-7-producing *E. coli* ABC133 plasmid pABC133-NDM found in 2012 in the United Arab Emirates (GenBank accession no. KX214671). The genomic environment surrounding $bla_{\text{NDM-12}}$ was $bla_{\text{NDM-12}}$ ble_{MBL} *trpF dsbC tnpA sulI qacEdeltaI* (type O in Fig. 2). This sequence showed greater than 99.9% identity with the pGUE-NDM plasmid (NCBI accession no. JQ364967) from *E. coli* strain GUE, which was isolated in India, as well as 99.9% identity with the pEC77-NDM plasmid (NCBI accession no. AB898038) from *E. coli* strain NCGM77, which emerged in Japan (14). The genomic environment surrounding $bla_{\text{NDM-13}}$ was *tnpA* IS*30* $bla_{\text{NDM-13}}$ ble_{MBL} *trpF dsbC cutA groES groL* (type P in Fig. 2); this sequence has been deposited in GenBank (accession no. LC012596). This structure, except for $bla_{\text{NDM-13}}$, was identical to that of pPMK1 expressed by *K. pneumoniae* PMK1 (from Nepal) (NCBI accession no. CP008933), the *Enterobacter hormaechei* CCHB10892 plasmid (from Brazil) (accession no. KF727591), pKPX-*1* from *K. pneumoniae* KPX (from Taiwan) (accession no. AP012055), and pNDM-MAR isolated from *K. pneumoniae* in Morocco (accession no. JN420336). PFGE and Southern hybridization analyses revealed that bla_{NDM}s in 19 isolates were found in the plasmids (Fig. S1).

There were no relationships among drug susceptibility profiles, drug resistance genes, MLSTs, and plasmid sizes.

To our knowledge, this is the first molecular epidemiological analysis of carbapenem-resistant *E. coli* clinical isolates in Nepal indicating that the isolates have various types of bla_{NDM}s. Five isolates belonging to ST648 in clade C were found, despite the lack of isolation of any carbapenem-resistant *E. coli* strain belonging to ST131 in medical settings in Nepal. CTX-M-producing *E. coli* ST131 and ST648 isolates have been reported to cause community-acquired infections in Nepal (15). The dissemination of ST648 *E. coli* isolates in various communities of Nepal appears to be advancing into medical settings, and ST648 *E. coli* isolates are acquiring carbapenem and aminoglycoside resistance.

In conclusion, carbapenem-resistant *E. coli* isolates producing NDMs and 16S rRNA methylases have been spreading in medical settings in Nepal. Gram-negative pathogens producing NDMs and 16S rRNA methylases are resistant to clinically important carbapenems (16) and aminoglycosides (17), respectively. Our previous studies revealed that Gram-negative pathogens, including *Acinetobacter baumannii* (18), *E. coli* (6), *K. pneumoniae* (19), and *Providencia rettgeri* (20), that produce NDMs and 16S rRNA methylases have emerged in medical settings in Nepal. Although this study addressed only carbapenem-resistant *E. coli* isolates, these results suggest the necessity of monitoring the dissemination of both aminoglycoside- and carbapenem-resistant *E. coli* strains in medical settings in Nepal.

This study was reviewed and approved by the Institutional Review Board of the Institute of Medicine at Tribhuvan University (reference no. 6-11-E). The study protocol was carefully reviewed and approved by the ethics committee of the National Center for Global Health and Medicine (reference no. 1268). Individual informed consent was waived by the ethics committee listed above because this study used currently existing samples collected during routine medical care and did not pose any additional risks to the patients. Patient information was anonymized and deidentified prior to the analysis. The study protocol was reviewed and approved by the Biosafety Committee, National Center for Global Health and Medicine (approval no. 28-M-053).

Nucleotide sequence accession number(s). The whole-genome sequences of all 38 isolates were deposited in GenBank as accession no. DRA005225 (for the experiment, accession no. DRX069506 to DRX069543; for the run, accession no. DRR075609 to DRR075646).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.01425-17.

SUPPLEMENTAL FILE 1, XLSX file, 0.1 MB.
SUPPLEMENTAL FILE 2, XLSX file, 0.1 MB.
SUPPLEMENTAL FILE 3, PDF file, 0.1 MB.

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